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## Evolutional Origin of Bacterial Glutamate Racemase

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Glutamate racemase (EC 5.1.1.3), an enzyme of microbial origin, shows significant sequence similarity with mammalian myoglobins, in particular in the regions corresponding to the E and F helices, which constitute the heme binding pocket of myoglobins. Glutamate racemase binds tightly an equimolar amount of hemin leading to loss of racemase activity. Although this enzyme shows sequence similarity with aspartate racemase, the latter does not bind hemin. Neither racemase has cofactors, but contain essential cysteine residues.

**Keywords:** Specific inhibition by hemin/ E and F helices of myoglobin/ Essential cysteine residues

D-Glutamate is an essential component of peptidoglycans of bacterial cell walls, and is produced from L-glutamate by glutamate racemase (EC 5.1.1.3) or from  $\alpha$ -ketoglutarate by D-amino acid aminotransferase (EC 2.6.1.21) [1]. Most amino acid racemases, such as alanine racemase (EC 5.1.1.1), require pyridoxal 5'-phosphate (PLP) as a coenzyme, and the racemase reaction is facilitated by formation of internal and external Schiff base intermediates. In contrast, a few other amino acid racemases, such as glutamate racemase [2] and aspartate racemase (EC 5.1.1.13) [3], are independent of any cofactor, and contain no carbonyl moieties or metals. Their reaction mechanisms have not been elucidated. We have cloned the glutamate racemase gene from *P. pentosaceus*, expressed it in *E. coli* and purified the enzyme to homogeneity [4]. The purified enzyme contains no co-factors, but does have essential cysteine residues.

Glutamate racemase showed considerable sequence similarity with aspartate racemase. Linear alignment of their sequences by introducing gaps to maximize identity revealed an overall similarity of 14%. However,

sequence similarity in the internal region (69–192 of the glutamate racemase sequence) was much higher; 31 of 124 residues being common. If the mutationally allowed substitutions for similar residues were taken into consideration, the similarity score increased to 68% in this region. In particular, the sequences around the two cysteine residues ( $^{74}\text{Cys}$  and  $^{184}\text{Cys}$  of glutamate racemase) were highly similar to each other. Both enzymes contain an essential cysteine residue as reported previously, suggesting that either  $^{74}\text{Cys}$  or  $^{184}\text{Cys}$ , or both play an essential role in catalysis.

Glutamate racemase shows also a high sequence similarity with bovine myoglobin among various proteins registered in the National Biomedical Research Foundation and the Swissprot protein sequence data-banks. The analogous region between glutamate racemase and myoglobin occur mainly in the region between  $^{46}\text{Phe}$  and  $^{150}\text{Gly}$  of bovine myoglobin which corresponds to the region from  $^{92}\text{Val}$  to  $^{183}\text{Gly}$  of glutamate racemase. Twenty-seven of the 92 residues of glutamate racemase are common to the corresponding residues of the myoglobin. The similarity score is 52%

### BIOFUNCTIONAL MOLECULES —Molecular Microbial Science—

#### Scope of research

*Structure and function of biocatalysts, in particular, pyridoxal enzymes and NAD enzymes are studied to elucidate the dynamic aspects of the fine mechanism for their catalysis in the light of recent advances in gene technology, protein engineering and crystallography. In addition, the metabolism and biofunction of selenium and some other trace elements are also investigated. Development and application of new biomolecular functions of microorganisms are also studied to open the door to new fields of biotechnology. For example, molecular structures and functions of thermostable enzymes and their application are under investigation.*



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in this region, if the similar residues of permissible mutational substitution are taken into account. The amino acid sequences of myoglobins from various sources are highly conserved. The abalone myoglobin shows high sequence similarity with human indoleamine 2,3-dioxygenase, but not with other myoglobins. We found no significant sequence similarity between the abalone myoglobin and glutamate racemase. Similarity scores between glutamate racemase and the other myoglobins were: 21–27% identity in the range of the 92 amino acid residues. Cyanobacterial myoglobin from *Nostoc commune* showed the lowest sequence similarity (21%) with glutamate racemase. Significant sequence similarities were also found between glutamate racemase and other globin family proteins such as hemoglobins in this same region. Bacterial hemoglobin from *Vitreoscilla* shows the lowest sequence similarity with glutamate racemase among the various hemoglobins examined.

Proteins analogous to bovine myoglobin in primary structure were also searched by means of the same data-banks. The sequence similarity is dependent on the kind of proteins and their sources: myoglobins from other sources, 38–85%;  $\alpha$  and  $\beta$ -chains of mammalian hemoglobins, 21–31%; *Vitreoscilla* hemoglobin, 24%; *N. commune* myoglobin, 16%; glutamate racemase, 26% (in the range between <sup>46</sup>Phe and <sup>150</sup>Gly of bovine myoglobin). Bovine myoglobin shows higher sequence similarity with glutamate racemase than prokaryotic myoglobin and hemoglobin. Aspartate racemase was also analogous to bovine myoglobin in the region from <sup>102</sup>Ile to <sup>196</sup>Gly corresponding to that from <sup>46</sup>Phe to <sup>150</sup>Gly of bovine myoglobin: 14 residues were common between the two proteins. However, this sequence similarity was much lower than that found between glutamate racemase and bovine myoglobin.

The analogous range (residue numbers, 46–150) of bovine myoglobin contains the regions corresponding to E and F helices, which constitute the heme binding pocket. E7 of the E helix of bovine myoglobin, <sup>64</sup>His, which is essential in binding molecular oxygen, is replaced by Gln in the bacterial myoglobin and the bacterial hemoglobin. An analogous Gln occurs as <sup>110</sup>Gln in glutamate racemase. Moreover, <sup>68</sup>Val of E11, which is highly conserved among globin family proteins, is also conserved as <sup>114</sup>Val. Accordingly, we examined the interaction of glutamate racemase and aspartate racemase with hemin. When the enzymes were assayed in the presence of various concentrations of hemin, only glutamate racemase was inhibited by hemin. The inhibition was concentration-dependent. A plot of reciprocal of glutamate racemase activity against hemin concentrations showed that hemin produced a mixed type inhibition. The  $K_i$  value for hemin was estimated to be about 3.7 mM from these data. When glutamate racemase was incubated with hemin at various concentrations, a stoichiometric complex was formed and isolated by gel filtration. However, no appreciable amount of hemin was bound with aspartate racemase under the same conditions. The complex of glutamate

racemase with hemin was reduced with dithionite. The ESR spectrum of the oxidized form resembled that of hemoglobin under the same conditions. Thus, glutamate racemase resembles hemoglobins in having a heme binding pocket, in which two nitrogen atoms of some amino acid residues are probably ligated to iron in the coordination complex with hemin. Hemin inhibits glutamate racemase either by binding near the active site or at some other site where the binding causes a conformational change of the active site.

Proline racemase, 4-hydroxyproline epimerase and diaminopimelate epimerase contain an essential cysteine residue, and show sequence similarity with each other in the moiety around the cysteine residues. These enzymes have been proposed to evolve from a common ancestral protein. Glutamate racemase as well as aspartate racemase also contains an essential cysteine residue, but shows no sequence similarity to these three enzymes. However, a high sequence similarity in the regions of two cysteine residues occurs between glutamate racemase and aspartate racemase. It is suggested that glutamate racemase and aspartate racemase have derived from a common evolutionary origin which is different from the common ancestor for proline racemase, 4-hydroxyproline epimerase and diaminopimelate epimerase.

The high sequence similarity between glutamate racemase and the globin family proteins, in particular myoglobins, and formation of its inactive equimolar complex with hemin, suggest that the enzyme may be derived from the evolutionary origin of globin family proteins. Aspartate racemase also may have evolved from the common ancestral protein, but its structure may have been altered more extensively than glutamate racemase by divergence. Lactic acid bacteria may have been producing glutamate racemase and aspartate racemase, namely globin family-like proteins, which diverged from an ancestral globin protein after the ability to synthesize hemin was lost. Alternatively, lactic acid bacteria inherently never produced hemin, and acquired from other organisms the gene for the globin family proteins, which then diverged to glutamate racemase and aspartate racemase. Whatever may be the case, glutamate racemase is the first proven microbial enzyme that is structurally similar to globin family proteins and to stoichiometrically bind hemin to form a catalytically inactive complex.

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